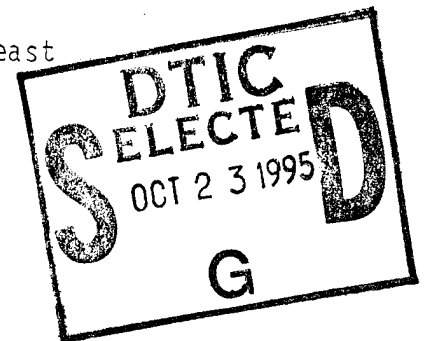


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FOREWORD

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Introduction

The mechanism and regulation of mammalian cell DNA replication is not fully understood. To date, several mammalian enzymes and proteins have been shown to be required for replication *in vitro* (reviewed in Kelly, 1988; Challberg and Kelly, 1989; Stillman, 1989; Hurwitz et al., 1990; Malkas et al., 1990a; Stillman et al., 1992). Many of these proteins were identified using a simian virus 40 (SV40) based *in vitro* DNA replication system (Li and Kelly, 1984) and include: DNA polymerase α -primase, DNA polymerase δ , proliferating cell nuclear antigen (PCNA), RP-A, RF-C or Activator 1 (A-1) protein complex and topoisomerases I, II. However, the manner in which these polypeptides, and those yet to be identified, associate with one another as well as their ability to act in concert to efficiently replicate DNA is not well defined.

There have been several reports regarding the isolation of large complexes of proteins for DNA synthesis from extracts of eukaryotic cells (reviewed in Mathews and Slabaugh, 1986; Fry and Loeb, 1986; Malkas et al., 1990a; Reddy and Fager, 1993). The first description of a multiprotein complex from human (HeLa) cells that fully supported replication of DNA *in vitro* was by Malkas et al., 1990b. The multiprotein form of the human cell DNA polymerase was further purified by Q-Sepharose chromatography and glycerol gradient sedimentation and was shown to fully support origin-specific and large T-antigen-dependent *in vitro* SV40 DNA replication (Malkas et al., 1990b; Applegren et al., 1995). We have also identified and characterized a 17S multiprotein form of DNA polymerase from murine (FM3A) cells (Wu et al., 1994) that is capable of supporting DNA replication using the polyoma (PyV)-based *in vitro* DNA synthesis system (Murakami et al., 1986; Dermody et al., 1988). The isolated mouse and HeLa cell multiprotein form of DNA polymerase was termed a **Multiprotein DNA Replication Complex (MRC)**. Furthermore, we proposed a model to represent the MRC that was isolated from murine and human HeLa cells based on the fractionation, sedimentation and chromatographic behavior of the replication proteins.

Most recently, we have purified and characterized an MRC from MDA MB 468 human breast cancer cells (Coll et al., in preparation). Similar to the HeLa cell MRC, the breast cell MRC possesses a sedimentation coefficient of 18S in a continuous sucrose gradient. The breast cell MRC fully supports the *in vitro* bidirectional replication of the SV40 genome in the presence of SV40 origin sequences and viral large T-antigen. Several of the DNA replication proteins comprising the MRC have been identified by Western blot analyses and enzymatic assays and include: DNA polymerase α -primase, DNA polymerase δ , PCNA, RP-A, RF-C and DNA topoisomerases I, II. Importantly, the DNA replication proteins that comprise the breast cell MRC demonstrate the same sedimentation and chromatographic profiles as those polypeptides composing the murine and HeLa cell MRC. We conclude that there is now sufficient evidence to suggest that DNA synthesis is mediated by a multiprotein complex in human breast cells. Further examination of the breast cell MRC will elucidate the mechanisms of breast cell DNA replication.

Furthermore, the *in vitro* DNA replication of the SV40 genome by the breast cell MRC may serve as a valuable system for analyzing the actions of anti-breast cancer agents that directly affect cellular DNA synthesis. The following points support this premise. First, the breast cell MRC mediates many of the steps of eukaryotic DNA replication. Second, use of the breast cell MRC allows the accurate assessment of whether an anti-breast cancer agent directly interferes

with the DNA replication process, while being uncoupled from other cellular processes that may secondarily affect DNA synthesis. Third, use of the breast cell MRC permits an investigator to examine the interaction of an anti-breast cancer agent with the whole DNA replication apparatus, not just individual DNA replication proteins.

We have recently reported on the interaction of the anticancer agent, camptothecin, with the human cell MRC (Coll et al., in press). Camptothecin is a plant alkaloid that traps topoisomerase I in a ternary (drug-DNA-enzyme) cleavable complex. Researchers speculate that collision of moving replication and transcription forks with cleavable complexes results in irreversible DNA single strand breaks. Little is known about camptothecin's cell-killing mechanism beyond cleavable complex formation. In our studies on the interaction of camptothecin with the human cell MRC, we found a close correlation between the camptothecin IC_{50} s (concentration of drug inhibiting process by 50%) for HeLa cell survival, intact HeLa cell DNA synthesis and MRC-mediated DNA synthesis. In addition, in the presence of increasing concentrations of camptothecin, we found a positive correlation between the extent of inhibition of MRC-mediated DNA synthesis and the formation of ternary cleavable complexes. Our data support the use of the MRC as a model system to study in detail the mechanism of action of camptothecin. As derivatives of camptothecin have demonstrated efficacy in treating patients with breast carcinomas, understanding the drug's mechanism of action may facilitate the development of improved anti-breast-cancer analogs.

Experimental Methods

(I) Purification and characterization of the breast cell MRC from MDA MB 468 human breast cancer cells

Cell culture

Suspension cultures of human breast (MDA MB 468) cells (Cailleau et al., 1978) were adapted from monolayer cultures. The cells were grown in Joklik's modified Eagle's medium supplemented with 5% each of irradiated calf and fetal bovine serum. Exponentially growing cells (5×10^5 cells/ml of medium) were harvested and washed three times with phosphate buffered saline (PBS): 20 mM Na_2HPO_4 , 0.15 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 . The cells were then pelleted at low-speed centrifugation (1000 rpm, 5 minutes, 4°C) and the cell pellets stored at -80°C until fractionation.

Cell fractionation and column chromatographic procedures

MDA MB 468 cells (20-30g cells) were homogenized and the breast cell MRC was purified essentially as described by Malkas et al. (1990b) and as outlined in figure A1. Sucrose gradient centrifugation was performed as described in Wu et al. (1994).

Enzyme assays

DNA polymerase α activity with activated calf thymus DNA templates was assayed according to published procedures (Malkas et al., 1990b). One unit of DNA polymerase activity equals 1 nmol of total 3H -TTP incorporated into DNA per hour at 35°C.

Topoisomerase I activity was assayed as described in Hickey et al. (1988) for the relaxation of pSVO⁺ plasmid DNA (Stillman et al., 1985).

Immunodetection of RF-C, PCNA, RP-A, primase and DNA polymerase δ

Denaturing polyacrylamide gel electrophoretic analysis of the various MDA MB 468 protein fractions was performed as previously described (Laemmli, 1970). Western blot analyses of these proteins were performed according to published procedures (Malkas et al. 1990b; Applegren et al., 1995). The protein quantity of MDA MB 468 fractions used in these analyses was 20 μ g. The polymerase δ polyclonal antibody, a generous gift from Dr. Marietta Lee, was prepared against the C-terminal portion of polymerase δ polypeptide. The anti-PCNA antibody was purchased from Boehringer-Mannheim. The anti-primase antibody was a generous gift from Dr. William Copeland. The anti-RF-C monoclonal antibodies (mAb-11 and mAb-19), which recognize the 140 kDa subunit of the RF-C protein complex, and the anti-RP-A antibody (p70) that recognizes the 70 kDa subunit of RP-A, were very generous gifts from Dr. Bruce Stillman. The appropriate species-specific horseradish peroxidase conjugated secondary antibodies were used in the immunoblots.

Purification of SV40 large T-antigen

SV40 large T-antigen was purified from infected cells according to our previously published procedures (Malkas et al., 1990b).

***In vitro* SV40 DNA replication assay**

Assay reaction mixtures (25 μ l) contained 80 mM Tris-HCl, pH 7.5; 7 mM MgCl₂; 1 mM DTT; 3-20 μ of protein fraction; 0.5-1.0 μ g of purified SV40 large T-antigen; 50 ng of plasmid pSVO⁺ containing an insert of SV40 replication-origin sequences (Stillman et al., 1985); 100 μ M each dTTP, dATP, dGTP; 200 μ M each rCTP, rGTP, UTP; 4 mM ATP; 25 μ M [³²P]dCTP; 40 mM creatine phosphate; 1 μ g of creatine kinase. The reaction was incubated for 2 hours at 35°C. The replication reaction assay products were processed using DE81 filter binding to quantify the amount of radiolabel incorporated into the replication products (Sambrook et al., 1989). One unit of SV40 replication activity is equal to the incorporation of 1 pmol of dNMP into SV40 replication origin containing plasmid DNA per 2 hours under the standard assay conditions.

(II) Study of the interaction of camptothecin with the human cell MRC

Cell culture and harvest

Suspension cultures of HeLa cells were grown in Joklik's modified Eagle's medium supplemented with 5% each irradiated calf and fetal bovine serum. Exponentially growing cells (5×10^5 cells/ml of medium) were harvested and washed 3 times with phosphate buffered saline; 8.4 mM Na₂HPO₄, 137 mM NaCl, 1.5 mM KH₂PO₄. The cells were then pelleted by low-speed centrifugation (200g, 5 min, 4°C). Cell pellets were stored at -80°C prior to initiating isolation of the MRC.

the MRC.

Fractionation and chromatographic scheme for isolation of human cell MRC

The HeLa cell MRC was isolated as described by Malkas et al. (1990b). The protein fraction designated Q-Sepharose peak, which contains the DNA replication-competent MRC, was used in the experiments described in this report.

HeLa cell-survival assays

HeLa cell-survival assays were performed in the presence of various concentrations of camptothecin, according to the procedure described by Glisson et al. (1984).

Measurement of intact HeLa cell DNA synthesis

Exponentially growing HeLa cells were incubated at 37°C with various concentrations of camptothecin in the presence of ³H-thymidine. After a one-hour incubation, cells were lysed (Horwitz et al., 1971) and the amount of radiolabel incorporated into DNA was determined by the isolation and counting of acid-insoluble material (Sambrook et al., 1989).

***In vitro* SV40 DNA replication assay**

Performed as described in section 1.

Topoisomerase I assay

Topoisomerase I activity was measured by incubating 0.3 µg supercoiled pSVO+ DNA with either 2 units purified topoisomerase I or 8 µg Q-Sepharose peak, in the absence or presence of various concentrations of camptothecin. Incubations were performed for 20 minutes at 37°C. Each reaction (15 µl) was stopped with 1% SDS and the DNA products resolved on a 1.0% agarose gel containing TAE buffer (40 mM Tris acetate, 2 mM EDTA). After ethidium bromide (1 µg/ml) staining of the gels (Sambrook et al., 1989), topoisomers were visualized with a u.v. light source.

5' end-labeling of linear plasmid DNA

pSVO+ DNA, 20 µg, was digested with 40 units Hind III for 60 minutes at 35°C. One µg of the digested DNA was incubated with 1 µl Klenow fragment (5 units/µl) for 30 minutes at room temperature in a reaction mixture containing: 2.5 µl end-labeling buffer (0.5 M Tris-HCl (pH 7.6), 0.1 M MgSO₄, 1 mM dithiothreitol, 500 µg/ml BSA); 0.75 µl 100 mM MgCl₂; 2.5 µl 10X dGTP; 2.5 µl 10X dATP; 2.5 µl 10X dTTP; 4 µl ³²P-dCTP (3000 Ci/mmol, 10 mCi/ml); 2.25 µl dH₂O. Reactions were stopped by the addition of 1 µl 0.5 M DTA and heating to 65°C for 5 minutes; reactions were then diluted to 100 µl with a buffer containing: 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Unincorporated deoxynucleotide triphosphates were removed by chromatography through a p60 gel filtration column (Sambrook et al., 1989).

SDS precipitation of topoisomerase I - cleavable complexes

Cleavage of DNA by human topoisomerase I was performed as follows. Briefly, 5 units of

purified topoisomerase I or 8 μ g Q-Sepharose peak, 1 μ l end-labeled pSVO+ DNA and various concentrations of camptothecin were added to a reaction buffer containing 10 mM Tris-HCl (pH 9.0), 0.5 mM EDTA and 10 μ g/ml BSA. After a 5-minute incubation at 37°C, SDS precipitation of double-stranded DNA topoisomerase I complexes was performed as described by Liu et al. (1983).

Results

I. Isolation and characterization of the breast cell MRC

Human breast cell DNA replication proteins co-fractionate as a readily sedimentable form.

To determine whether the human breast cell MRC could be isolated in a readily sedimentable form, as had been previously observed for the MRC isolated from HeLa cells (Malkas et al., 1990b; Applegren et al., 1995) and mouse FM3A cells (Wu et al., 1994), we subjected human breast MDA-MB 468 cells to the fractionation scheme outlined in figure 1. The PEG NE/S-3, S-4 and P-4 fractions were collected and assayed for DNA polymerase α activity, as well as for the presence of other DNA replication proteins and enzymes.

The majority of the DNA polymerase α enzymatic activity isolated from the MDA-MB 468 cells was observed to partition with the sedimentable P-4 fraction following PEG precipitation of the NE/S-3 fraction from the human breast cell homogenate (Table 1). This pattern of partitioning for the DNA polymerase α enzymatic activity had been observed previously during the isolation of the MRC from HeLa cells (Malkas et al., 1990b; Applegren et al., 1995) and mouse FM3A cells (Wu et al., 1994).

DNA polymerase δ (Byrnes et al., 1976; Lee et al., 1984) has been shown to be required for the synthesis of SV40 replication origin containing DNA *in vitro* (Prelich et al., 1987). To determine whether the DNA polymerase δ polypeptide was associated with the DNA polymerase enriched human breast P-4 fraction Western blot analyses of the human breast cell derived PEG NE/S-3, S-4 and P-4 fractions were performed using a polyclonal antibody prepared against the C-terminal peptide of DNA polymerase δ (Yang et al., 1992). These analyses clearly demonstrate the presence of this DNA replication essential enzyme in the PEG NE/S-3 fraction (figure 2). In addition, it was observed that the DNA polymerase δ polypeptide is exclusively associated with the human breast P-4 fraction (figure 2).

Western analyses of the human breast cell protein fractions demonstrated that the 59 kDa polypeptide subunit of DNA primase (figure 2) is exclusively associated with the human breast cell derived P-4 fraction. The DNA replication protein RF-C (Tsurimoto and Stillman, 1989; Lee et al., 1991) was also found to co-purify with P-4 fraction (figure 2). This was determined by immunoblot analyses using a monoclonal antibody that recognizes the 140 kDa subunit of the RF-C protein complex. RF-C polypeptide was observed to partition exclusively with the P-4 fraction.

PCNA polypeptide was also detected in the P-4 fraction (figure 2), but unlike the DNA polymerases α and δ , DNA primase, and RF-C, this protein is found in the S-4 fraction as well. This result suggests that the PCNA polypeptide may not be tightly associated with the DNA polymerases, DNA primase, and RF-C. Alternatively, the relative abundance of PCNA, relative

to the other proteins, might influence its distribution during the fractionation.

Western blot analyses were also performed to determine whether RP-A (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988) associates with human breast P-4 fraction (figure 2). Monoclonal antibody to the 70 kDa subunit of the RP-A protein was used in these studies. These experiments show that RP-A fractionates with the P-4 fraction. RP-A was also found in the S-4 fraction. These results, like those obtained for the PCNA polypeptide, suggest that only a fraction of these cellular proteins co-purify with the human breast P-4 fraction.

The co-fractionation of the human breast cell DNA polymerases α and δ , DNA primase, RF-C, RP-A, and PCNA proteins with the P-4 fraction suggests that these activities are associated in a readily sedimentable form.

The human breast cell MRC present in the readily sedimentable P-4 fraction supports the *in vitro* replication of DNA.

Since the human breast cell DNA polymerases α and δ , DNA primase, RF-C, RP-A, and PCNA proteins partition with the readily sedimentable P-4 fraction, an attempt was made to determine whether this fraction could support the *in vitro* replication of DNA. This was assessed by determining whether the P-4 fraction was efficient for the *in vitro* replication of the SV40 replication origin containing DNA (table 1). It was observed that the human breast PEG NE/S-3 fraction is fully competent to support SV40 DNA replication *in vitro* (table 1). A DE81 filter binding analysis of the ^{32}P -dTTP radiolabeled DNA replication products formed in the reaction showed that the highest level of ^{32}P -dTTP incorporation into DNA occurred when the PEG NE/S-3 fraction was incubated in the presence of the SV40 large T-antigen (table 1). A negligible amount of ^{32}P -dTTP radiolabel was incorporated in those replication reactions lacking the SV40 large T-antigen (table 1). When the human breast PEG NE/S-3 fraction was subfractionated into the S-4 and P-4 fractions the ability to support *in vitro* large T-antigen dependent SV40 replication was found to reside exclusively with the sedimentable P-4 fraction (table 1). These data indicate that all of the activities required to carry out large T-antigen dependent SV40 DNA replication are associated with the human breast sedimentable P-4 fraction.

Further purification of the human breast cell MRC

The human breast cell MRC present in the P-4 fraction was further purified by Q-Sepharose chromatography. This method of chromatography was successfully used for the further purification of the HeLa cell MRC (Malkas et al., 1990b; Applegren et al., 1995). The Q-Sepharose elution profile of the human breast cell derived P-4 fraction DNA polymerase activity is shown in figure 3. The DNA polymerase activity was observed to eluate from the Q-Sepharose column as an initial sharp peak, with a small broad shoulder of activity eluting at higher salt concentrations. Little DNA polymerase activity was observed in the column flow through or wash fractions (data not shown).

The peak of DNA polymerase activity that eluted from the Q-Sepharose column (Q-Sepharose peak) was examined by both Western blotting and activity measurements to determine whether other DNA replication essential proteins co-purified with the polymerase. Based on our previous work on the purification of the HeLa cell MRC (Malkas et al., 1990b; Applegren et al., 1995), we decided that the Q-Sepharose peak fraction should be comprised of the pool of column

presence of the different replication proteins.

Western blot analysis demonstrates that DNA polymerase δ was found to exclusively co-purify with the DNA polymerase α activity in Q-Sepharose peak fraction (figure 2). The DNA polymerase δ polypeptide was not detectable in the Q-Sepharose FT fraction. Like DNA polymerase δ , the RF-C and DNA primase polypeptides were also observed to co-purify with the Q-Sepharose peak fraction but were not observed in the FT fraction (figure 2). Western blot analyses also show that both RP-A and PCNA co-elute with the Q-Sepharose peak protein fraction (figure 2). However, PCNA and RP-A were also found in Q-Sepharose flow-through fraction.

We observed that topoisomerase I activity co-elutes with the DNA polymerase α activity following Q-Sepharose chromatography of the P-4 fraction (figure 4). Supercoiled plasmid DNA incubated in the absence of protein is shown in lane 5. A conversion of supercoiled plasmid DNA into relaxed form II DNA was observed following its incubation with the Q-Sepharose peak (lane 3) and the Q-Sepharose flow-through (lane 2). Furthermore, the activity was inhibited by camptothecin, a specific inhibitor of topoisomerase I (lane 4) (Hsiang et al., 1985). Like RP-A and PCNA, it appears that only a fraction of the total cellular topoisomerase I co-purifies with the MRC-enriched protein fraction.

The form of human breast cell MRC in the Q-Sepharose peak fraction is replication competent.

Since the human breast cell DNA polymerases α and δ , DNA primase, RF-C, RP-A, topoisomerase I, and PCNA proteins co-chromatograph with the DNA polymerase α activity in the Q-Sepharose peak fraction, we determined whether this fraction could support *in vitro* SV40 DNA replication (table 2). The human breast cell derived Q-Sepharose peak and FT fractions were each assayed for *in vitro* SV40 DNA replication activity. Only the Q-Sepharose peak fraction was observed to contain SV40 DNA replication activity. The Q-Sepharose FT fractions supports little SV40 DNA synthesis.

The breast cell MRC contained in the Q-Sepharose peak exhibits a sedimentation coefficient of 18S.

The HeLa cell MRC sediments at 18-21S in sucrose and glycerol density gradients (Malkas et al., 1990b; Applegren et al., 1995), while the mouse FM3A MRC has a sedimentation coefficient of 17S (Wu et al., 1994). To determine the sedimentation coefficient of the human breast cell MRC we subjected the replication-competent Q-Sepharose peak fraction to velocity sedimentation analysis in a 10-30% sucrose gradient containing 0.5M KCl. The resulting sucrose gradient fractions were analyzed for DNA polymerase activity (figure 5). The relative position of the Q-Sepharose peak DNA polymerase in the sucrose gradients was estimated by comparing the migration distances of the polymerase activities through the gradients to that of marker proteins (Malkas et al., 1990b; Wu et al., 1994; Applegren et al., 1995). The breast cell MRC present in the Q-Sepharose peak fraction was observed to sediment with an S-value of 18S. This 18 S-value of the replication-competent human breast cell MRC would account for its ready sedimentation to the sucrose interphase (P-4 fraction) following the centrifugation of the PEG NE/S-3 fraction

(figure 1).

II. Interaction of camptothecin with the human cell MRC

Inhibition of HeLa cell-survival, intact HeLa cell DNA synthesis, and MRC-mediated DNA synthesis by camptothecin

We performed cell survival assays to verify that camptothecin affects the ability of mammalian cells to proliferate, as reported by others (Andoh et al., 1987). We observed that 0.015 μ M camptothecin inhibited HeLa cell survival by 50%; at higher concentrations of the drug, no cells were visible following trypan blue dye staining of the tissue culture plates (Figure 7). This result is consistent with previously published work (Andoh et al., 1987) in which camptothecin cytotoxicity was examined in human lymphoblastic leukemia cell cultures.

Topoisomerase I plays a pivotal role in DNA replication, facilitating replication fork migration by unwinding positive supercoils as they accumulate ahead of the fork. The necessity of topoisomerase I in DNA synthesis is underscored by the sensitivity of intact HeLa cell DNA synthesis to low concentrations of camptothecin. Figure 8 shows that 3 H-thymidine incorporation into DNA by exponentially growing HeLa cells is inhibited by 50% at approximately 0.12 μ M. The occurrence of low levels of DNA replication at higher drug concentrations suggests that topoisomerase II may partially substitute for the function normally provided by topoisomerase I, as proposed by others (Annunziato et al., 1989).

Similarly, a low concentration of camptothecin was observed to inhibit the ability of the MRC to support *in vitro* DNA replication. *In vitro* SV40 DNA replication assays incubated with various concentrations of camptothecin were analyzed for total 32 P-dCTP incorporation into DNA. MRC-mediated DNA replication was inhibited by 60% when reactions contained 0.050 μ M camptothecin (Table 3). The results presented in Table 3 are typical of those obtained during this set of experiments. The relatively close correlation between the concentrations of camptothecin inhibiting DNA synthesis in intact cells and in the MRC-driven *in vitro* reactions supports the potential role of the MRC as a meaningful model system for the study of camptothecin and other anticancer agents that directly affect DNA synthesis.

Effect of camptothecin on MRC-associated topoisomerase I activity

We performed topoisomerase I assays to establish that the topoisomerase I activity present in the MRC is fully able to produce the hallmark ladder of DNA intermediates while converting a form I supercoiled plasmid DNA into a form II DNA (Figure 9a, lane 6). The pattern of topoisomers produced by the MRC topoisomerase I is indistinguishable from that generated by the purified topoisomerase I enzyme (Figure 9b, lane 7).

In order to examine the effect of camptothecin on MRC topoisomerase I activity, we performed topoisomerase I assays in the presence of various concentrations of the drug. In these assays, inhibition of topoisomerase I activity by camptothecin results in the accumulation of form I DNA. We observed an extensive level of inhibition of the MRC-topoisomerase I activity by camptothecin (Fig. 9a, lanes 1-5); as little as 0.050 μ M camptothecin caused a significant accumulation of form I DNA. From these experiments, we determined that 0.050 μ M

camptothecin inhibited MRC-topoisomerase I activity over 50%. This concentration of camptothecin is comparable to that which inhibits both *in vitro* and intact cell DNA synthesis by 50%; supporting the premise that the inhibition of DNA replication by camptothecin results from the drug's inhibition of topoisomerase I activity. The activity of the purified topoisomerase I enzyme was inhibited strongly by the same concentration range of camptothecin (Figure 9b, lanes 2-6).

SDS precipitation of camptothecin-stabilized cleavable complexes

Camptothecin inhibits topoisomerase I activity by trapping the enzyme in a reversible enzyme-drug-DNA cleavable complex, the formation of which leads to replication fork arrest and DNA fragmentation (Tsao et al., 1993). Studies reveal that precipitation of the cleavable complex by protein denaturants yields DNA single strand breaks and results in the covalent linkage of topoisomerase I to the 3' end of the breaks via a phosphotyrosine bond (Hsiang et al., 1985). Utilizing 5' end-labeled substrate DNA, the relative amount of cleavable complexes stabilized by camptothecin can be quantified via the isolation and counting of radioactive topoisomerase I-linked DNA breaks (Liu et al., 1983). Figures 10a and 10b show the precipitation of purified and MRC-associated topoisomerase I-DNA cleavable complexes in the presence of various concentrations of camptothecin. We observed an increase of cleavable complex formation as a function of camptothecin concentration for both the purified topoisomerase I enzyme and the MRC-associated protein (Figures 10a, 10b). The increase in cleavable complex formation correlates with our observation regarding the loss of topoisomerase I activity at higher concentrations of camptothecin (Figure 10a, lanes 1-5).

DISCUSSION

We have identified and purified a multiprotein complex from the human breast cell line MDA-MB 468. Thus far, the proteins and enzymatic activities identified with the human breast cell multiprotein complex include DNA polymerases α and δ , DNA primase, PCNA, RF-C, RP-A, and DNA topoisomerase I. Most importantly, we have demonstrated that this complex of proteins is fully competent to replicate SV40 DNA *in vitro*. This result suggests that all of the cellular protein activities required for *in vitro* SV40 DNA synthesis are present within the multiprotein complex isolated from human breast cells, and that these proteins are organized into a DNA synthesis complex. The MRC was further purified by chromatography on Q-Sepharose. The components of the MRC were observed to co-elute together from the column. In addition, it was determined that the purified complex has a sedimentation coefficient of 18S in velocity sedimentation analyses. The requirements for SV40 DNA replication *in vitro* by the isolated human breast cell 18S MRC are comparable to the requirements that have been observed with crude extracts from permissive cells (Li and Kelly, 1984); namely, the initiation of SV40 replication is dependent on the presence of both the SV40 large T-antigen and functional SV40 replication origin sequences.

Based on the fractionation and column chromatographic profiles for the individual proteins observed to be present in the MRC isolated from HeLa and mouse FM3A cells, we proposed a model to represent the mammalian MRC (Wu et al., 1994; Applegren et al., 1995). The model is

depicted in figure 6 and now includes the human breast cell MRC. The majority of DNA polymerases α and δ , primase, and RF-C are observed to co-fractionate from cells, and to co-elute following column chromatography. This would suggest that these proteins are fairly "tightly" associated within the MRC. We propose that these proteins form the "core" of the MRC. It is interesting to note that these proteins also function primarily in the elongation phase of DNA synthesis (Kornberg and Baker, 1992). Unlike the proteins that compose the MRC core, PCNA is observed to be more "loosely" associated with the MRC. Although PCNA co-fractionates and co-elutes, following column chromatography, with the MRC core proteins it is also found in fractions that do not support *in vitro* DNA synthesis. Because of these observations, PCNA, which functions as an accessory factor for DNA polymerase δ (Tan et al., 1986), is represented in our model as associated with the MRC, but not as a member of the MRC core complex. Furthermore, we are currently performing experiments to assay for the presence of topoisomerase II in the breast cell MRC; this protein has been identified as a component of the HeLa and murine cell MRCs.

The role played by large T antigen in the initiation of *in vitro* SV40 DNA replication has been recently reviewed (Borowiec et al., 1990; and Fanning and Knippers, 1992). Briefly, the large T antigen binds sites within the SV40 origin and melts the early palindrome region within the DNA. The addition of topoisomerase I and RP-A facilitates the further melting of the SV40 DNA. A similar mechanism can be envisioned for the initiation events occurring in *in vitro* SV40 DNA replication. We have observed that topoisomerase I and RP-A (data not shown), like PCNA, do not "tightly" associate with the MRC components that compose the MRC core. We, therefore, propose that topoisomerase I and RP-A constitute the MRC's "initiation" components. Together, the core and initiation components would constitute the mammalian MRC.

Since SV40 is extensively dependent on the cell's DNA synthetic machinery for its own DNA replication, our results indicate that the isolated MRC may play a role not only in SV40 DNA synthesis but in human breast cell DNA replication as well. The further study of the human breast cell MRC is expected to yield a clearer understanding of the role of this complex in mediating the many reactions required to carry out human breast DNA replication. This seems especially true since the MRC was isolated as an intact and functional entity from the cell, and therefore, presumably contains all of the components required to successfully replicate DNA. In addition, because the process of DNA replication is an important regulatory point for modulating cell proliferation, the elucidation of the role played by the human breast MRC, and its components, in this regulatory process will further our understanding of both normal and aberrant cell proliferation. Therefore, it is our goal to fully characterize this complex with respect to its structural components and activity, and relate this information to the higher level of organization within the cell.

Finally, our data support the use of the human cell MRC as a novel model system to study the anticancer agent, camptothecin. First, the concentration of camptothecin required to inhibit MRC mediated *in vitro* DNA replication correlates with that for intact HeLa cell DNA synthesis. Secondly, MRC-intact topoisomerase I activity was inhibited 50% at 0.05 μ M camptothecin. This concentration correlates with that required for the inhibition of *in vitro* and intact cell DNA replication, supporting the premise that camptothecin inhibits nucleic acid synthesis by selectively targeting topoisomerase I. Finally, the increased formation of MRC-intact topoisomerase I DNA

cleavable complexes at higher concentrations of camptothecin is consistent with the more pronounced inhibition of MRC-intact topoisomerase I activity by higher concentrations of the drug. Our results suggest that camptothecin interacts with the intact cell topoisomerase I and the MRC-associated topoisomerase I in a similar manner. Perhaps the greater sensitivity displayed by HeLa cells to camptothecin in the cell survival assays results from prolonged induction of the G₂ cell cycle arrest by long-lived DNA double strand breaks (Ryan et al., 1994) or the drug's disruption of RNA and protein synthesis (Horwitz et al., 1971).

Several aspects of camptothecin cytotoxicity remain to be investigated; for example, exact determination of the camptothecin DNA binding site as well as uncovering the lethal cellular events that occur beyond cleavable complex formation. We believe that some of these questions can be answered by employing the MRC as a model system to study the effects of camptothecin on DNA replication. We anticipate that future studies with the MRC will facilitate a greater understanding of camptothecin's mechanism of action and contribute pertinent information required for the development of analogs with improved anticancer activity.

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FIGURE LEGENDS

Figure 1. The flow diagram of the isolation scheme used to purify the human breast cell multiprotein DNA replication complex (MRC). A detailed description of the isolation scheme is presented in Malkas et al., (1990b) and Applegren et al., (1995).

Figure 2. Western blot analysis of the MDA-MB 468 derived protein fractions. Thirty micrograms of each protein fraction (NE/S-3, S-4, P-4, Q-Sepharose peak (QS), Q-Sepharose flow-through (FT), and sucrose gradient peak (SG)) were size fractionated on 8% polyacrylamide gels. The proteins were transferred to nitrocellulose filter membranes. The polypeptides DNA polymerase δ , DNA primase, PCNA, RF-C and RP-A were visualized by sequentially incubating the membranes with the appropriate primary antibodies, followed by species-specific secondary antibodies conjugated to horseradish peroxidase (HRP). Light-enhanced chemiluminescence detection of the immobilized protein was accomplished using the ECL Western blotting detection system (Amersham). The resolution profile of the ECL protein molecular weight markers is shown in the panel designated (M).

Figure 3. Q-Sepharose chromatographic profile of the MDA-MB 468 derived P-4 fraction. A description of the column preparation and elution conditions are provided in the text, Malkas et al. (1990b) and Applegren et al. (1995).

Figure 4. Topoisomerase I activity in the MDA-MB 468 derived protein fractions. This figure shows the degree of conversion of a supercoiled Form I plasmid DNA to a relaxed open circle Form II DNA following the incubation of .25 μ g of supercoiled plasmid DNA (pSVO+) with 8 μ g of Q-Sepharose peak (QS), Q-Sepharose flow-through (FT) and sucrose gradient peak (SG) protein fractions. The position of the supercoiled DNA (Form I) and the relaxed open circular DNA (Form II) are indicated. The degree of relaxation correlates directly with the level of topoisomerase I activity present in the protein fraction. The reaction was incubated at 37°C for 20 minutes, and then stopped by the addition of 1% SDS. The DNA topoisomers were resolved by electrophoresis in a 1.2% agarose gel containing TAE buffer (40 mM Tris acetate, 2 mM EDTA). The resolved DNA topoisomers were visualized by staining the gel with a solution of 1 μ g/ml ethidium bromide. Lane 5 shows the position of the supercoiled (Form I) pSVO+ DNA in the absence of the protein fraction. The degree of relaxation to Form II DNA by the topoisomerase I activity found to co-sediment in the sucrose gradient peak fraction is shown in Lane 1. Lane 2 represents conversion to relaxed Form II DNA found in the Q-Sepharose flow-through fraction. Lane 3 shows the conversion of the Form I DNA to the relaxed Form II by the topoisomerase I activity present in the Q-Sepharose peak fraction. Lane 4 demonstrates the inhibition of the Q-Sepharose peak topoisomerase I activity by 200 μ M camptothecin, a specific inhibitor of topoisomerase I activity (Hsiang et al., 1985).

Figure 5. Sucrose density gradient sedimentation analysis of the Q-Sepharose peak

fraction derived from MDA-MB 468 cells. 0.5 ml of the pooled Q-Sepharose column fractions containing the peak of in vitro SV40 DNA replication activity was layered onto a 9 ml 10-30% sucrose gradient containing 0.5 M KCl. The conditions for centrifugation and gradient fractionation were performed as described in Wu et al., (1994). The assay for DNA polymerase activity was performed as described in the Malkas et al. (1990b). The units of activity are as defined in Malkas et al. (1990b).

Figure 6. A proposed model for the organization of the identified protein components of the human breast cell MRC. A description of the model is discussed in the body of this report.

Figure 7. Effect of camptothecin on HeLa cell survival. 5×10^4 HeLa cells were seeded onto 60 mm cell culture plates and incubated for 24 hours at 37°C. Cells were then exposed for one hour to a control level of DMSO containing various concentrations of camptothecin: 0, 0.005, 0.05, 0.5, 5, 50 μ M. Control plates contained DMSO alone. The number of colonies formed in the presence of DMSO were within 5% of those formed in the absence of solvent. After removal of drug from cell cultures, the cells were rinsed twice with warm phosphate buffered saline and then incubated in fresh drug-free media for an additional 10-14 days. Colonies were visualized by staining with trypan blue dye. This graph represents the average of 2 independent experiments; bars represent deviations from the average. For those symbols apparently lacking error bars, the deviation from the average was contained within the symbol.

Figure 8. Effect of camptothecin on intact HeLa cell DNA synthesis. 5×10^4 HeLa cells were seeded onto 60 mm cell culture plates and incubated for 24 hours at 37°C. The cells were then labeled with 3 H-thymidine (1 μ Ci/ml of medium) and exposed to a constant level of DMSO containing various concentrations of camptothecin: 0, 0.1, 1, 5, 10, 20, 100 μ M. Plates containing DMSO alone served as controls. After a one hour incubation, cells were lysed and the level of DNA synthesis was measured by the isolation and counting of acid-insoluble material. This graph represents the average of 4 experiments; error bars represent standard error of the mean.

Figures 9a, 9b. Inhibition of MRC-associated topoisomerase I activity by camptothecin. (A) Reaction assays containing 8 μ g of the Q-Sepharose peak were incubated for 15 minutes at 37°C with 0.3 μ g supercoiled pSVO+ and various concentrations of camptothecin. Reactions were stopped by the addition of 1% SDS and topoisomers were resolved on a 1% agarose gel. After ethidium bromide (1 μ g/ml) staining of the gels, topoisomers were visualized by illuminating gels with a u.v. light source. Lane 7 shows the position of supercoiled pSVO+ DNA in the presence of DMSO. Lane 6 shows the conversion of supercoiled DNA to a relaxed, open circle form II DNA by the MRC-associated topoisomerase I present in the Q-Sepharose peak. Lanes 1-5 show the inhibition of MRC-topoisomerase I activity by increasing concentrations of camptothecin. **(B)** Topoisomerase I assays were performed as described in (A) using 2 units of purified topoisomerase I. Lane 1 shows the position of supercoiled pSVO+ in the presence of DMSO. Lane 7 shows the conversion of supercoiled DNA to relaxed form II DNA by purified topoisomerase I. Lanes 2-6 show the inhibition of topoisomerase I activity by increasing

concentrations of camptothecin.

Figures 10a, 10b. SDS precipitation of camptothecin-topoisomerase I cleavable complexes.

(A) Reaction assays containing 5 units of purified topoisomerase I and 1 ng of 5' end-labeled pSVO+ DNA were incubated with various concentrations of camptothecin for 5 minutes at 37°C. Reactions were stopped by the addition of a solution containing 2% SDS, 2 mM EDTA and 0.5 mg/ml sheared salmon sperm DNA (Liu, et al., 1983). After SDS precipitation of topoisomerase I-DNA cleavable complexes, pellets were transferred to vials containing scintillation cocktail and counted. The data presented are an average of two independent experiments; error bars represent the deviation from the average. **(B)** SDS precipitation assays were performed as described in part (A) using 8 µg MRC. The data are an average of three independent experiments; error bars represent the standard error of the mean.

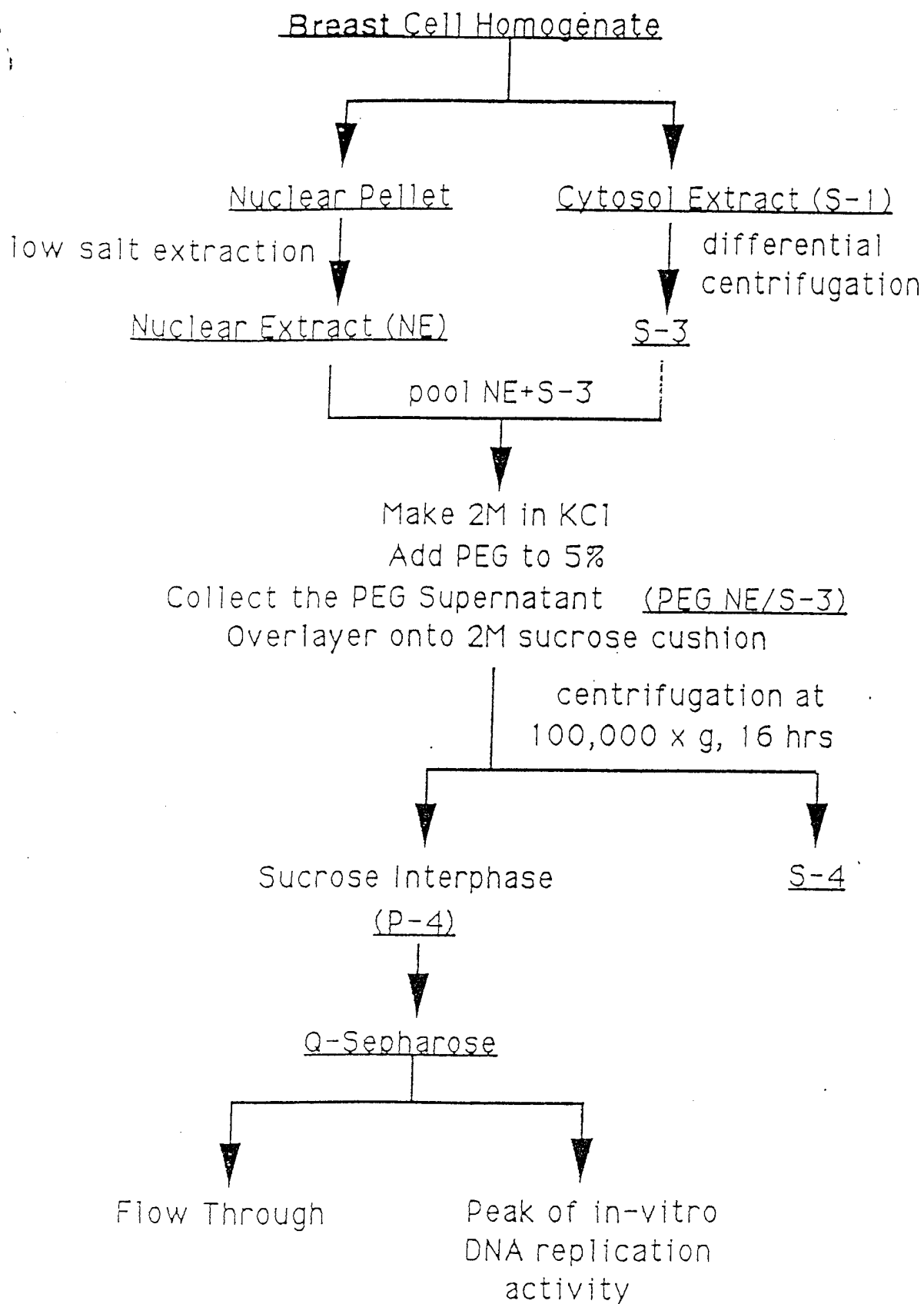


Figure 1

Western Blot Analysis of DNA Replication Proteins Present in the Breast Cancer Cell MRC

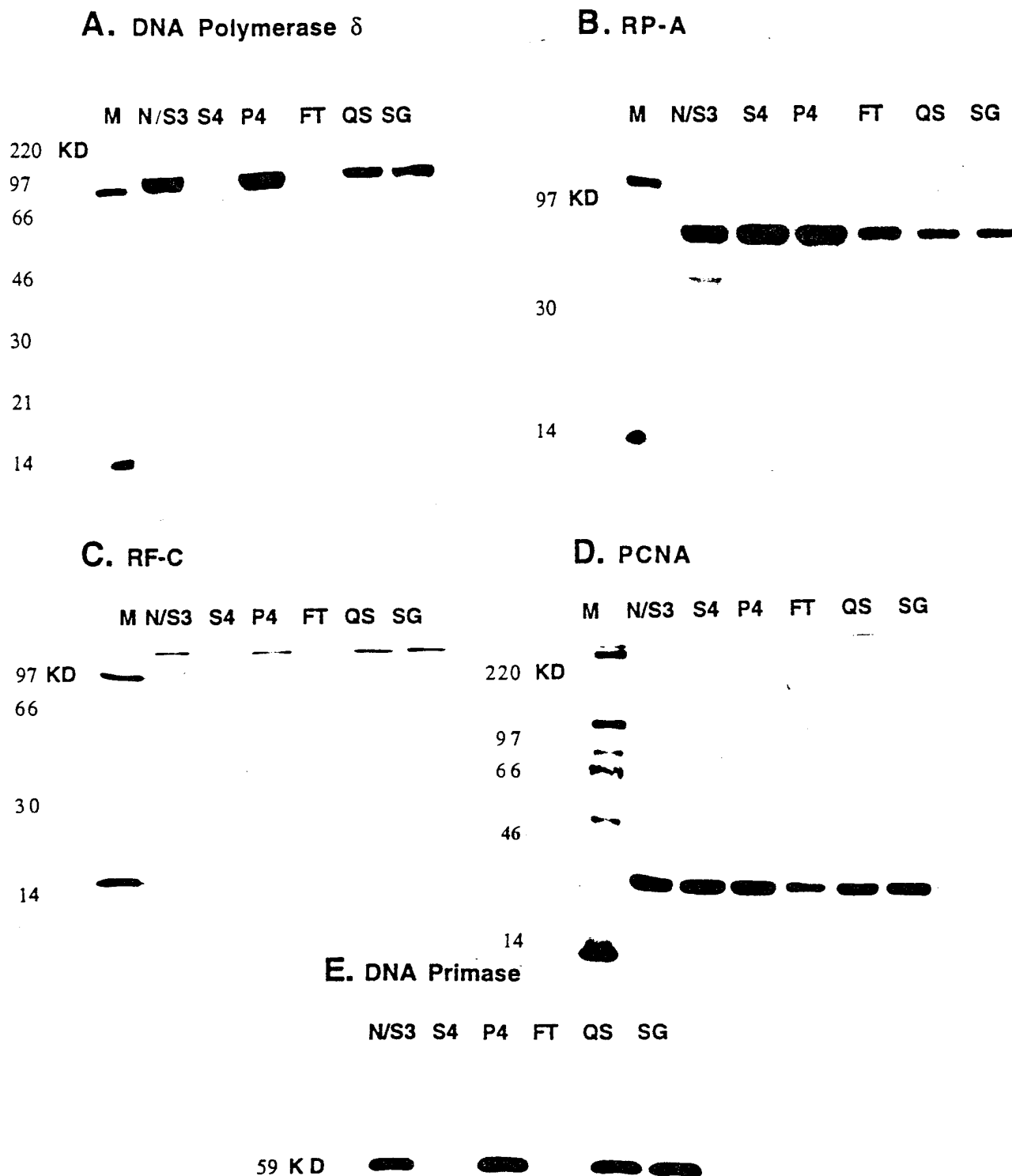


Figure 2

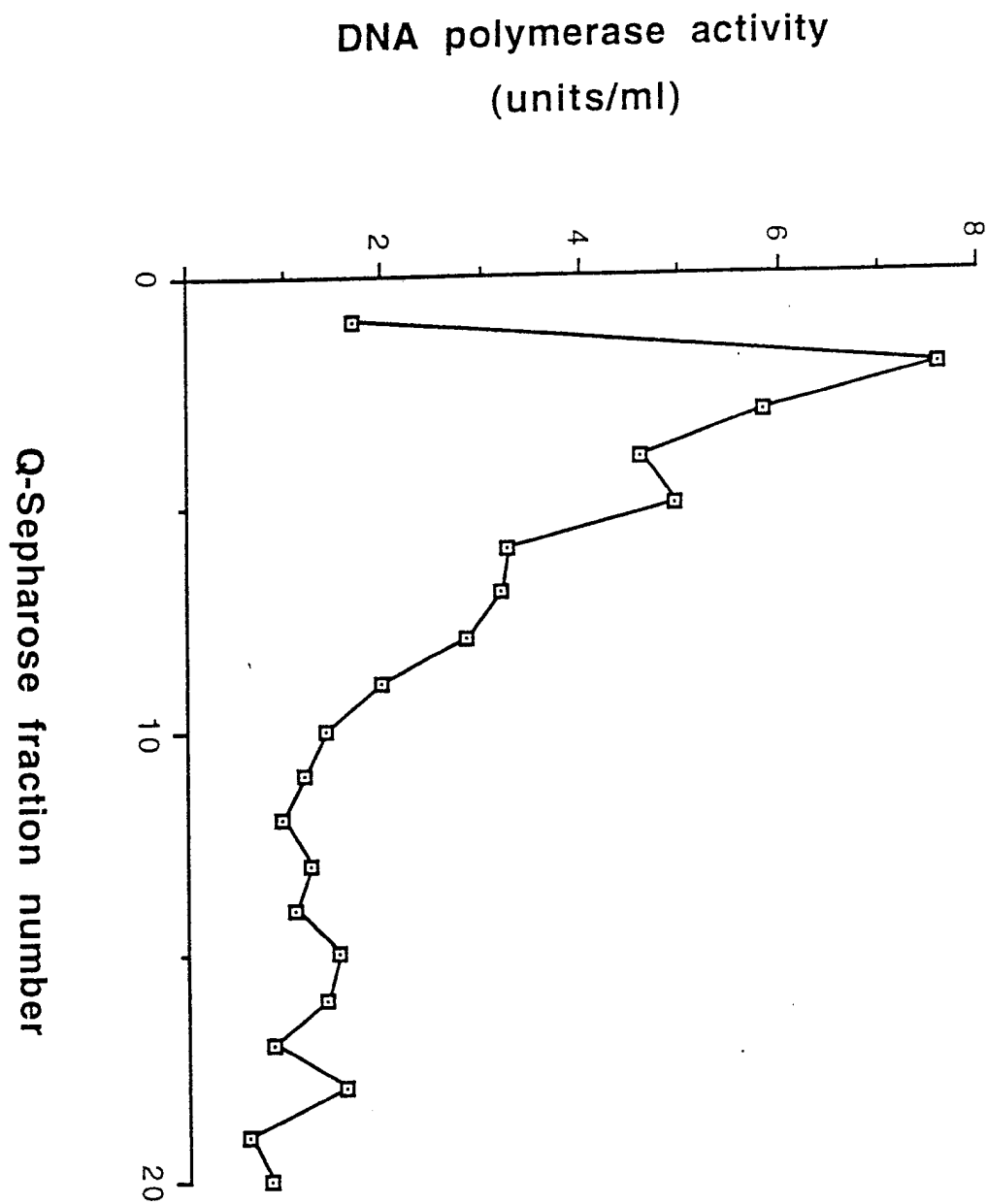


Figure 3

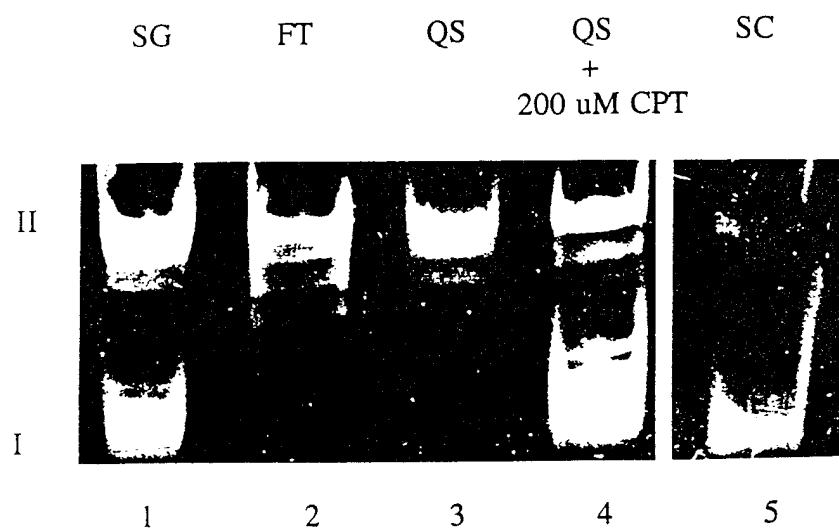


Figure 4

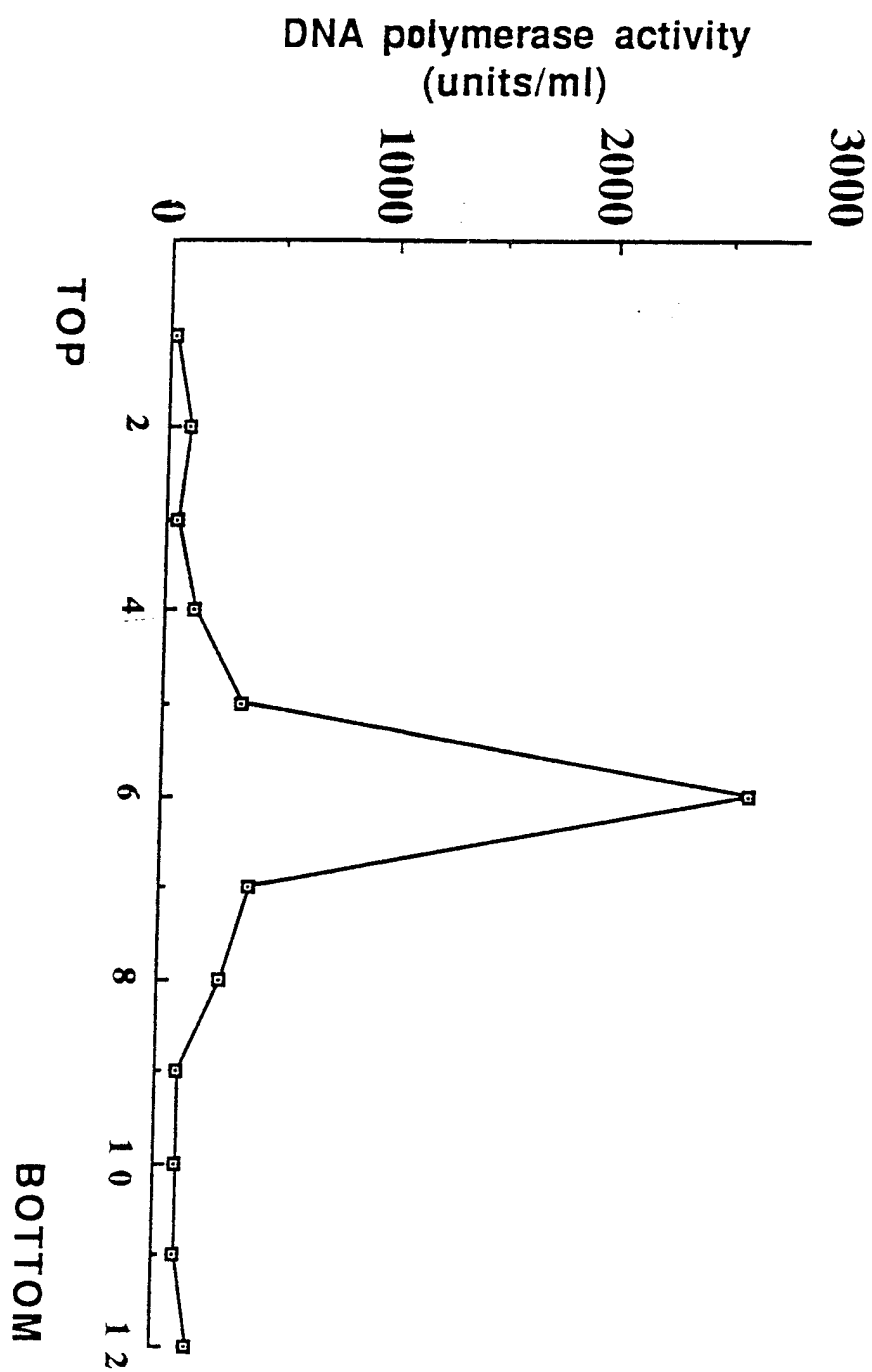


Figure 5

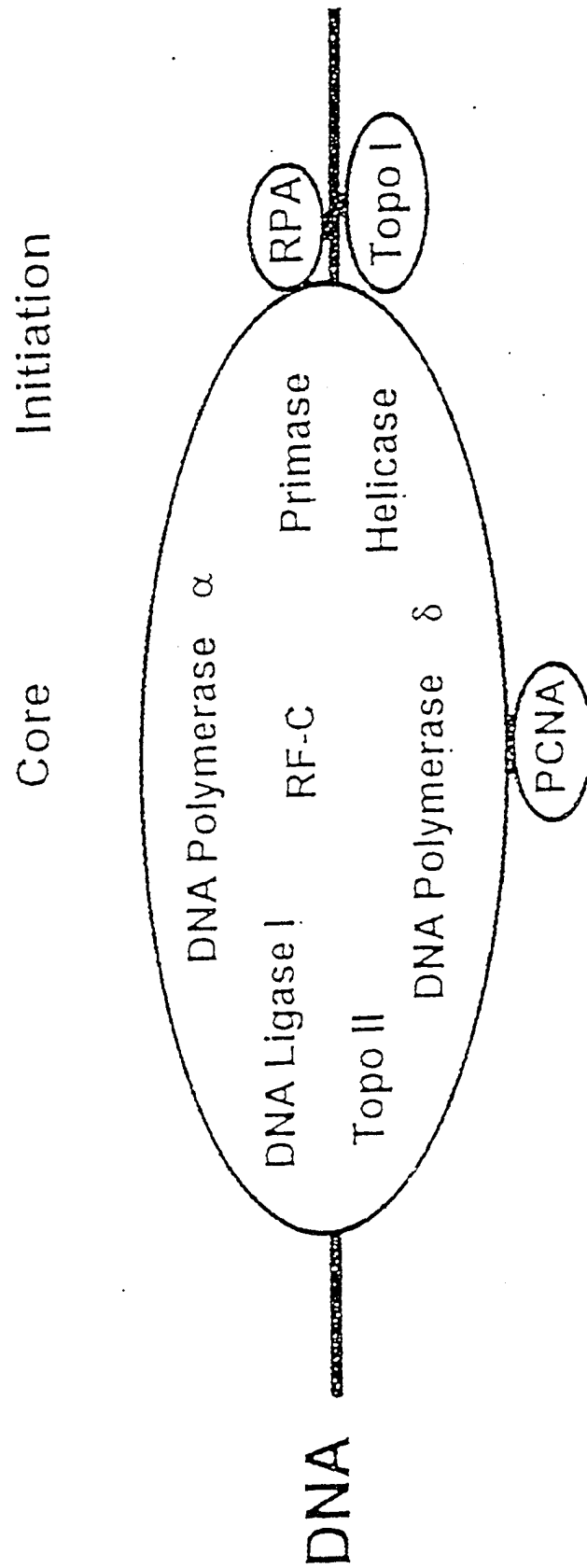


Figure 6

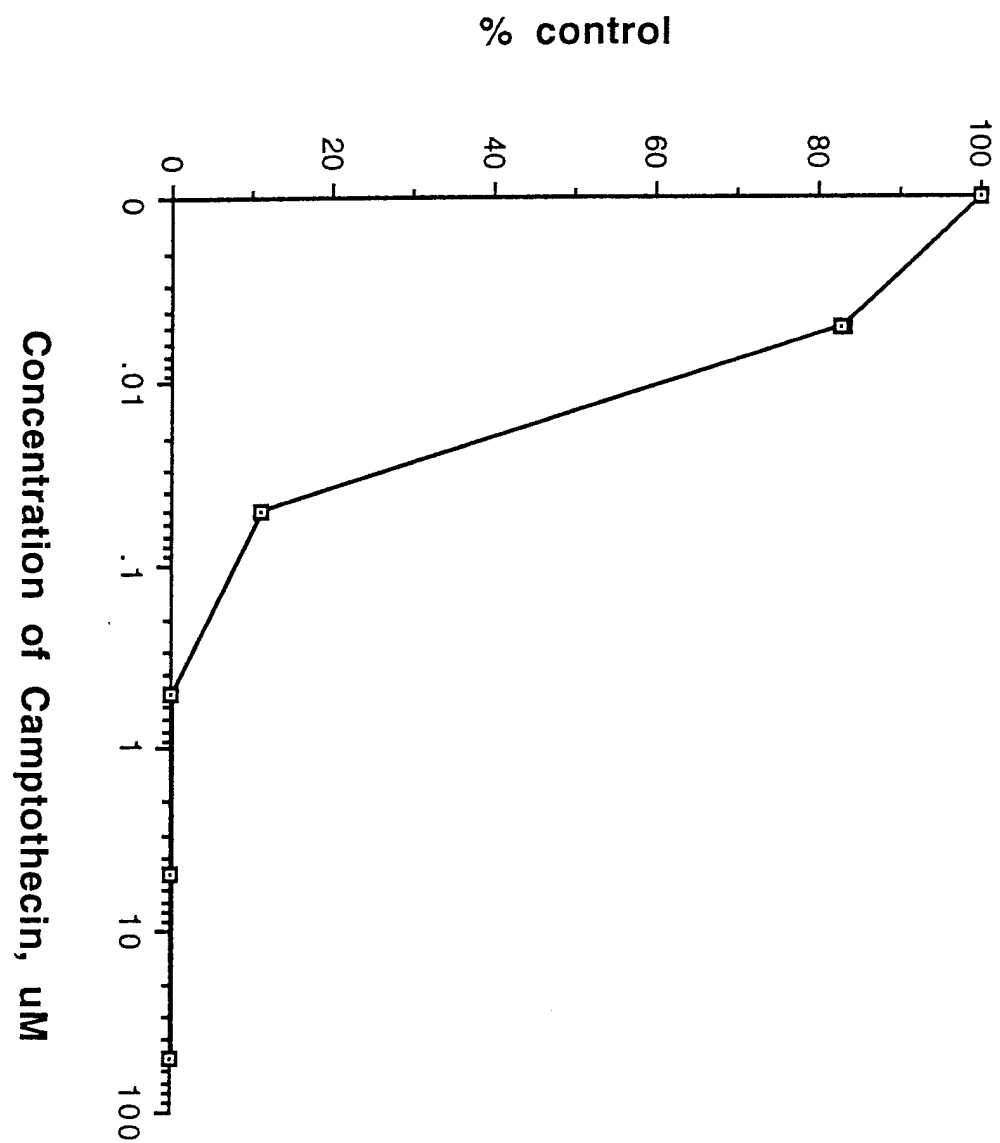


Figure 7

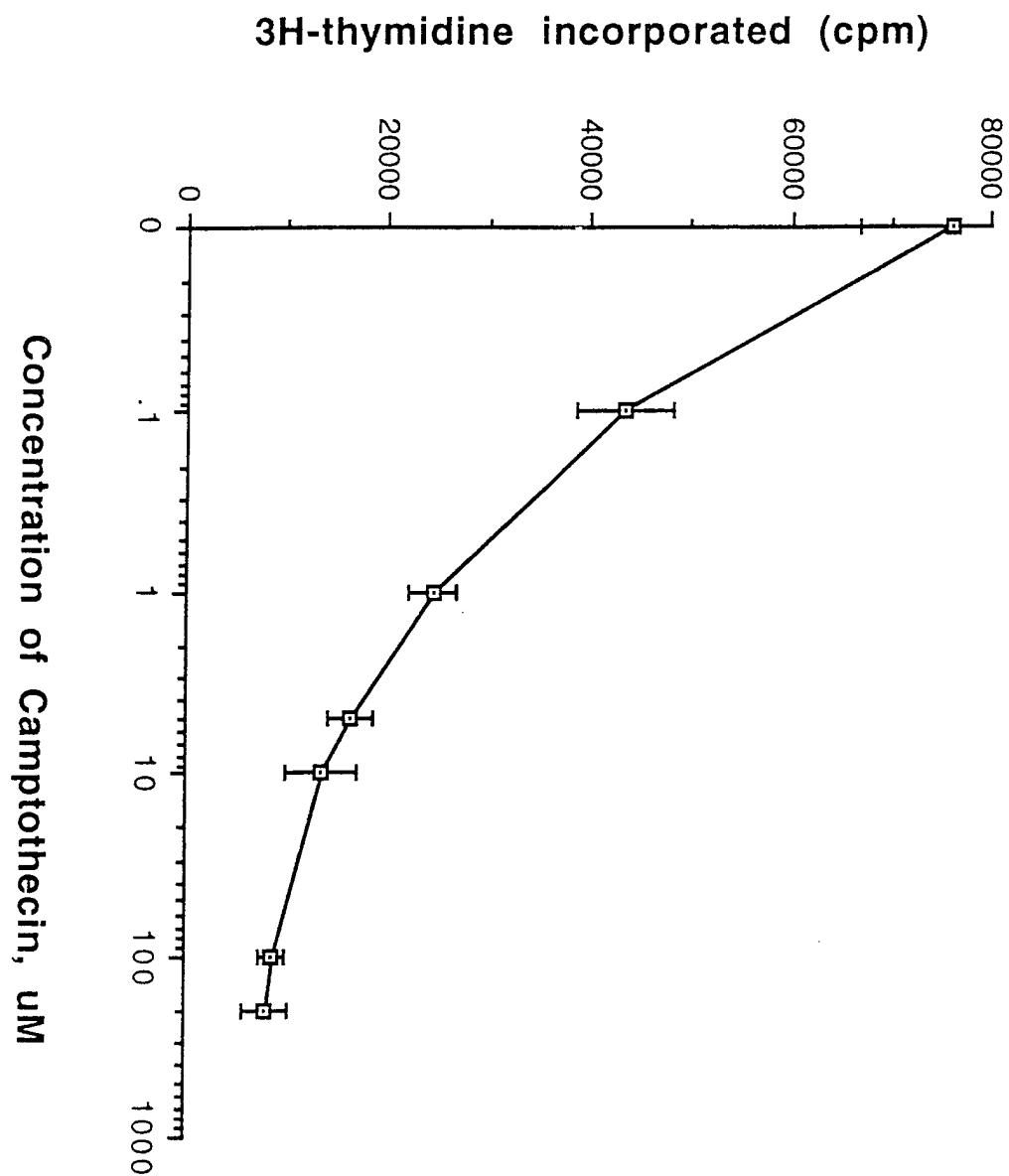


Figure 8

Camptothecin, μM

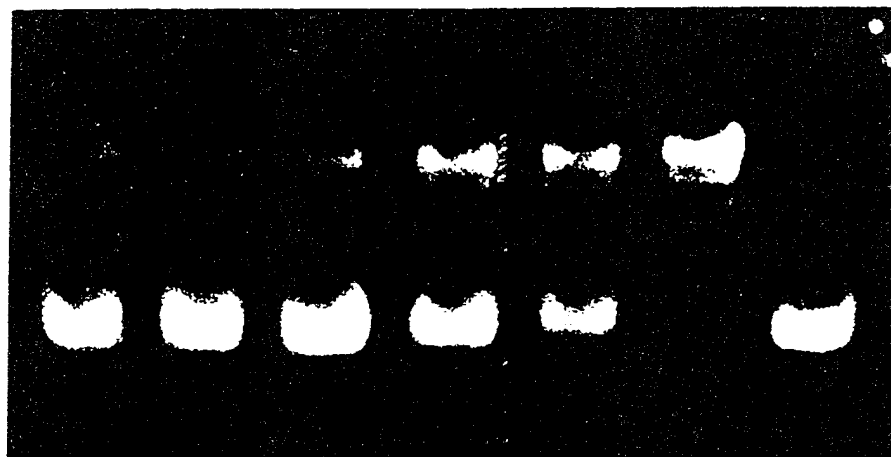
9a

→ II

→ I

Lanes

500 50 5 .5 .05 0 SC



1 2 3 4 5 6 7

Camptothecin, μM

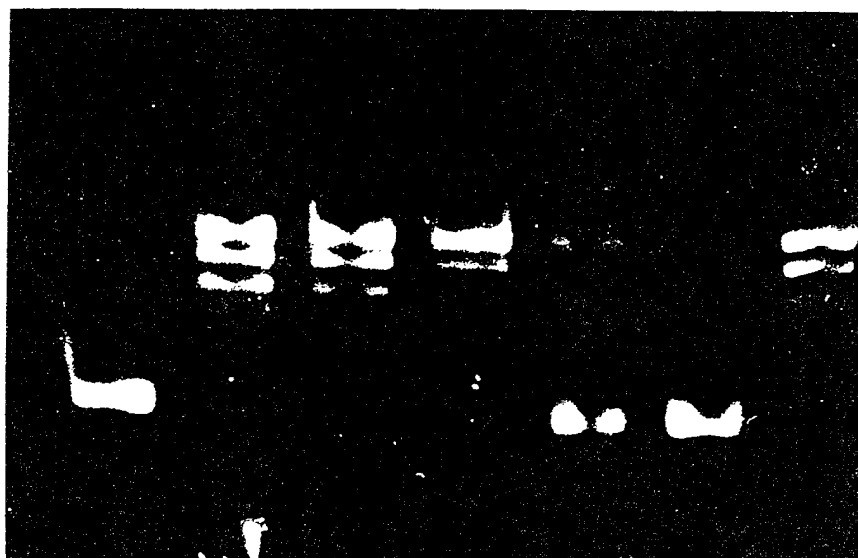
9b

→ II

→ I

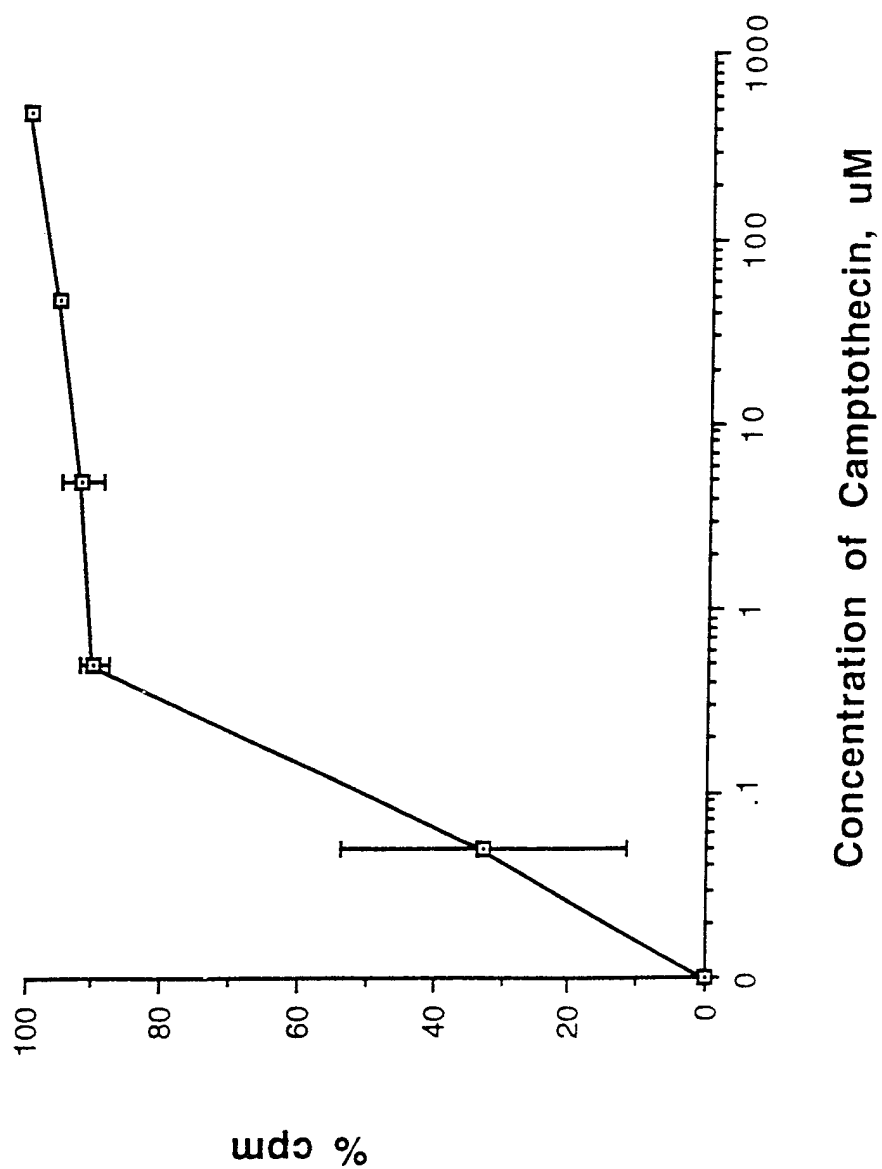
Lanes

SC .05 .5 5 50 500 0



1 2 3 4 5 6 7

Figures 9a, 9b



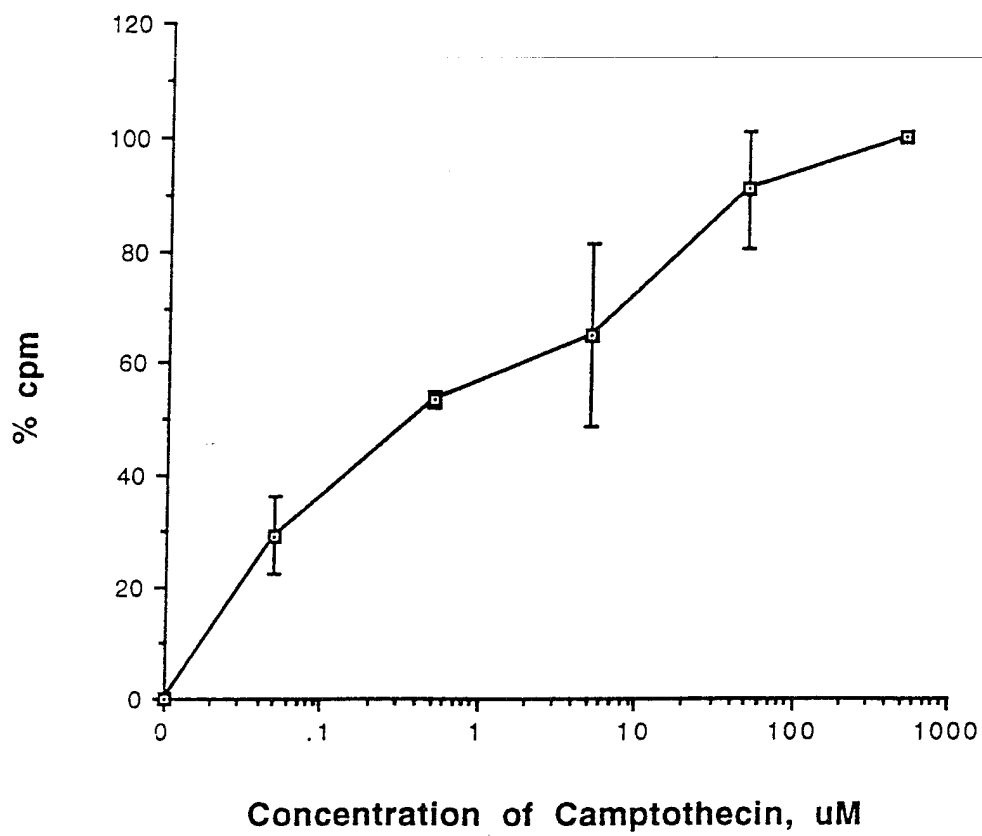


Figure 10b

Table 1. DNA polymerase α and *in vitro* DNA replication activities fractionate with the P-4 fraction.

FRACTION	PEG NE/S-3	S-4	P-4
DNA Polymerase α *	132.5	0.3	188.3
DNA Replication(+T)#	103.8	8.8	110.6
DNA Replication (-T)#	3.1	0.0	0.2

*DNA polymerase activity with activated calf thymus DNA templates was assayed according to published procedures (Malkas et al., 1990b). One unit of DNA polymerase activity equals 1nmole of ^3H -TMP incorporated into DNA per hour at 35°C. These values represent the average of three experiments.

#*In vitro* SV40 DNA replication assays were performed as described by Malkas et al., 1990b. One unit of SV40 replication activity is equal to the incorporation of 1pmole of $^{32}\text{dCMP}$ into SV40 origin containing DNA per 2 hours at 35°C. These values represent the average of three experiments.

Table 2. *In vitro* DNA replication activities fractionate with the Q-Sepharose Peak.

FRACTION	Q-Sepharose Peak	Flow Through (FT)
DNA Replication (+T)[#]	136.6	30.4
DNA Replication (-T)[#]	8.5	1.8

[#]*In vitro* SV40 DNA replication assays were performed as described by Malkas et al., 1990b. One unit of SV40 replication activity is equal to the incorporation of 1pmole of ³²PdCTP into SV40 origin containing DNA per 2 hours at 37°C. These values represent the average of three experiments.

Concentration Camptothecin, uM	³² P-dCTP Incorporated into DNA (cpm)	% Inhibition
0 uM	36,387	0
.05 uM	15,049	59
.5 uM	14,315	61
5 uM	11,343	69
50 uM	3,822	89

Table 3

MRC-driven in vitro SV40 DNA replication assays were performed in the presence of a constant amount of DMSO containing various concentrations of camptothecin (0, .05, .5, 5, 50, 500 uM). Reactions containing DMSO alone served as a control. The amount of ³²P-dCTP incorporated into DNA replication assay products was determined using DE81 filter binding.